

10/016, 481

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PCT

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 38/17, 39/38	A1	(11) International Publication Number: WO 99/24055 (43) International Publication Date: 20 May 1999 (20.05.99)										
<p>(21) International Application Number: PCT/SG98/00087</p> <p>(22) International Filing Date: 3 November 1998 (03.11.98)</p> <p>(30) Priority Data: 9703972-1 6 November 1997 (06.11.97) SG</p> <p>(71) Applicant (for all designated States except US): NATIONAL UNIVERSITY OF SINGAPORE [SG/SG]; 10 Kent Ridge Crescent, Singapore 119260 (SG).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): GOPALADRISH-NAKONE, Ponnampalam [SG/SG]; Industry & Technology Relations Office (INTRO), National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260 (SG). PU, Xiao, Chun [CN/CA]; Industry & Technology Relations Office (INTRO), National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260 (SG). WONG, Peter, Tsun-Hon [SG/SG]; Industry & Technology Relations Office (INTRO), National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260 (SG). GWEE, Mathew, Chnong, Eng [SG/SG]; Industry & Technology Relations Office (INTRO), National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260 (SG). KINI, R., Manjunatha [IN/SG]; Industry & Technology Relations</p>	<p>Office (INTRO), National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260 (SG).</p> <p>(74) Agent: APPLIED RESEARCH CORPORATION; Kent Ridge, P.O. Box 1016, Singapore 911101 (SG).</p> <p>(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p>Published <i>With international search report.</i></p>											
(54) Title: THERAPEUTIC MOLECULES												
<table border="1"> <caption>Latency (s) Data</caption> <thead> <tr> <th>Group</th> <th>Latency (s)</th> </tr> </thead> <tbody> <tr> <td>S l.v.</td> <td>~10.5</td> </tr> <tr> <td>P l.v.</td> <td>~15.5*</td> </tr> <tr> <td>S p.o.</td> <td>~10.0</td> </tr> <tr> <td>P p.o.</td> <td>~17.5*</td> </tr> </tbody> </table>			Group	Latency (s)	S l.v.	~10.5	P l.v.	~15.5*	S p.o.	~10.0	P p.o.	~17.5*
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<p>(57) Abstract</p> <p>The present invention relates generally to peptide molecules and to derivatives, homologues, analogues and mimetics thereof capable of inducing or facilitating analgesia or partial analgesia alone or in combination with other analgesic molecules. The present invention also contemplates a method of inducing or facilitating analgesia or partial analgesia by the administration of a peptide or a derivative, homologue, analogue or mimetic thereof. The amino acid sequence of the peptide molecules of the present invention are derived from or based on amino acid sequences of snake venom toxins, and, in particular, α-neurotoxins.</p>												

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THERAPEUTIC MOLECULES

FIELD OF THE INVENTION

The present invention relates generally to peptide molecules and to derivatives, homologues, analogues and mimetics thereof capable of inducing or facilitating analgesia or partial analgesia alone or in combination with other analgesic molecules. The present invention also contemplates a method of inducing or facilitating analgesia or partial analgesia by the administration of a peptide or a derivative, homologue, analogue or mimetic thereof. The amino acid sequence of the peptide molecules of the present invention are derived from or based on amino acid sequences of snake venom toxins, and, in particular, α -neurotoxins.

BACKGROUND OF THE INVENTION

The increasing demand for improved and more efficacious analgesics is providing the incentive for the pharmaceutical industry to consider new approaches for identifying and designing new therapeutic molecules. One source considered to be of potential interest in the quest for new pharmaceutical agents is the identification of molecules from the natural environment including molecules from life forms in the environment. Much effort is now being spent, therefore, on screening aquatic environments, river beds, coral, plants, microorganisms and higher animals for potentially useful molecules. This search is often referred to as "natural product screening". The level of biodiversity in the Asian and Australasian regions provides a particularly useful target for natural product screening.

Reptiles are one of the oldest life forms on earth of which snakes form a particularly interesting group. Many venomous snakes have evolved toxin molecules such as α -neurotoxins which are highly effective in inducing a range of neurological dysfunctions.

Whereas snake venom has been studied to the extent of producing antidotes and antivenene, snakes have not been extensively studied as a potential source of therapeutic molecules.

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In work leading up to the present invention, the inventors studied snake venom from a particularly venomous snake, the King cobra (*Ophiophagus hannah*). This snake is broadly distributed in India, Burma, Thailand, Malaysia, Indochina, Southern China, Indonesia, Japan and the Philippines (Tin *et al*, 1991). To date, six α -neurotoxins have
5 been isolated from the king cobra venom (Joubert, 1973; Chang *et al*, 1993; Song *et al*, 1994). Little, however, is known of the pharmacological properties of those toxins.

The inventors have now surprisingly determined that peptide molecules having an amino acid sequence or derived from or based on portions of snake venom toxins have analgesic properties.

10 SUMMARY OF THE INVENTION

One aspect of the present invention provides a peptide comprising a sequence of amino acids having at least 60% similarity to a sequence of amino acids from a portion of a toxin from snake venom and which peptide is capable of inducing or facilitating analgesia or a derivative, homologue, analogue or mimetic of said peptide.

15 Another aspect of the present invention relates to a peptide comprising a sequence of amino acids having at least 60% similarity to an amino acid sequence from a portion of an α -neurotoxin from venom from a snake of the family of snakes selected from *Elapidae*, *Viperidae*, *Colubridae* or *Crotalidae* and which peptide is capable of inducing or facilitating analgesia or a derivative, homologue, analogue or mimetic of said peptide.

20 Yet another aspect of the present invention is directed to a peptide comprising the amino acid sequence N P F P T [SEQ ID NO:1] and which peptide is capable of inducing or facilitating analgesia or a derivative, homologue, analogue or mimetic of said peptide.

Still yet another aspect of the present invention provides a peptide having the amino acid sequence

25 N P F P T X₁ X₂ K R X₃ X₄ [SEQ ID NO:2]

wherein X₁, X₂, X₃ and X₄ may be the same or differed and each is any amino acid residue wherein said peptide is capable of inducing or facilitating analgesia or a

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derivative, homologue, analogue or mimetic of said peptide.

In yet another aspect of the present invention, there is provided a peptide having an amino acid sequence selected from the list consisting of:

N P F P T W R K R P [SEQ ID NO:3];

5 N P F P T R K R P [SEQ ID NO:4];

N P R P T W R K R P [SEQ ID NO:5]; and

N P F P T W K R K H [SEQ ID NO:6];

or a derivative, homologue, analogue or mimetic of one or more of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:5 or SEQ ID NO:6 and wherein said peptide is capable of
10 inducing or facilitating analgesia.

The present invention also provides a composition comprising a peptide as defined above alone or in combination with one or more pharmaceutically acceptable carriers and/or diluents and/or one or more other active molecules such as an analgesic molecule or compound.

15 Still another aspect of the present invention contemplates a method of inducing or facilitating analgesia said method comprising administering a peptide as hereinbefore defined for a time and under conditions sufficient to induce pain relief.

Even yet another aspect of the present invention is directed to the use of a peptide as hereinbefore defined in the manufacture of a medicament for inducing or facilitating
20 analgesia.

The present invention further extends to antibodies to the peptides as hereinbefore defined.

The peptides of the present invention are referred to as "analgesic" peptides.

Throughout this specification, unless the context requires otherwise, the word "comprise",
25 or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

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Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

Sequence Identity Numbers (SEQ ID NOs.) for the amino acid sequences referred to in the specification are defined following the bibliography.

- 5 Single and three letter abbreviations used throughout the specification are defined in Table 1.

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TABLE 1

Single and three letter amino acid abbreviations

	Amino Acid	Three-letter Abbreviation	One-letter Symbol
5	Alanine	Ala	A
	Arginine	Arg	R
	Asparagine	Asn	N
10	Aspartic acid	Asp	D
	Cysteine	Cys	C
	Glutamine	Gln	Q
	Glutamic acid	Glu	E
	Glycine	Gly	G
15	Histidine	His	H
	Isoleucine	Ile	I
	Leucine	Leu	L
	Lysine	Lys	K
	Methionine	Met	M
20	Phenylalanine	Phe	F
	Proline	Pro	P
	Serine	Ser	S
	Threonine	Thr	T
	Tryptophan	Trp	W
25	Tyrosine	Tyr	Y
	Valine	Val	V
	Any residue	Xaa	X

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is graphical representation of typical tracings showing that the addition of 10 μ g of analgesic peptide had no effect on the contractile (twitch) responses of the chick biventer muscle evoked by electrical field stimulation (25V, 5Hz x 10s, 1 ms pulse width, every 100s).

Figure 2 is a graphical representation showing the hot-plate latencies before (open bar) and 3h after (hatched bar) administration of the analgesic peptide i.v. (72ng/g) or p.o. (360ng/g). Data represent mean values of latency time \pm SEM. *Significantly different from control, $p < 0.05$, Student's two-tailed t-test.

Figure 3 is a graphical representation showing the hot-plate latencies before (open bar) and 3 h after (hatched bar) administration of the analgesic peptide alone (P) or with naloxone together (P+N) or with L-NAME together (P+L). Data represent mean values of latency time \pm SEM. The peptide was given at 72ng/g (N) or 360ng/g (p.o.) with naloxone (6mg/kg) or L-NAME (20ng/kg). *Significantly different from before administration, $P < 0.05$, Student's two-tailed t-test.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a peptide or a derivative, homologue, analogue or mimetic thereof which comprises an amino acid sequence which is at least 60% similar to a sequence of amino acids in a toxin and, more particularly, an α -neurotoxin from snake venom and which peptide is capable of inducing or facilitating analgesia. The peptide is conveniently, in isolated or purified form.

The term "analgesia" is used in its broadest sense to include any form of pain relief ranging from complete removal of pain to a reduced the level of pain. The pain relief may be considered a reduced sensibility to pain without loss of consciousness. The pain relief may be localized or systemic. Pain includes acute pain, sharp localised pain, more centralized pain and any other form of sensation which contributes to discomfort. An "analgesic" is considered herein to be a molecule which induces analgesia.

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The subject of the analgesia is generally a warm blooded animal or bird such as but not limited to a human, primate, livestock animal (eg. sheep, cow, horse, donkey, pig), companion animal (eg. dog, cat), laboratory test animal (eg. mouse, rat, guinea pig, rabbit, hamster), captive wild animal (eg. deer, fox), caged bird (eg. parrot) and poultry
5 bird (eg. chicken, duck, pheasant, goose, turkey). Preferably, the subject is a human, primate or other warm blooded mammal. Most preferably, the subject is a human.

The term "peptide" encompasses a proteinaceous molecule comprising from about 2 amino acid residues to about 50 amino acid residues. Preferably, the peptide comprises from about 3 amino acid residues to about 40 amino acid residues. Even more
10 preferably, the peptide comprises from about 4 amino acid residues to about 30 amino acid residues. Particularly preferred embodiments include peptides comprising from about 4 amino acid residues to about 20 amino acid residues such as from 4 to 15 and 4 to 10 amino acid residues. Although the amino acid sequence of the analgesic peptides is derived from or based on an α -neurotoxin from snake venom, it does not extend to the
15 full length α -neurotoxin but rather only to a part thereof.

The analgesic peptide may be glycosylated or unglycosylated and/or may contain a range of other molecules fused, linked, bound or otherwise associated to the peptide such as amino acids, lipids, carbohydrates or other peptides, polypeptides or proteins. Reference
20 hereinafter to a "peptide" includes a peptide comprising a sequence of amino acids as well as a peptide associated with other molecules such as amino acids, lipids, carbohydrates or other peptides, polypeptides or proteins. For the purposes of the present description, a molecule comprising a sequence of at least about 50 amino acids is regarded as a peptide.

The analgesic peptides of the present invention may be produced by chemical synthetic
25 techniques or may be produced by recombinant DNA technology as discussed further below. The peptides may also be fragments of larger α -neurotoxin molecules from snake venom. The fragments may be naturally occurring fragments or generated by the action of proteases, peptidases, amidases, lysins or other enzymes as well as by sonic disruption, heat, chemical disruption and/or shearing.

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The amino acid sequence of the analgesic peptides of the present invention are considered to be derived from or based on a snake venom toxin and, more particularly, α -neurotoxin. This is not to imply, however, that the preferred peptides are necessarily derivatives of naturally occurring snake venom toxins since particularly useful peptides are, in accordance with the present invention, chemically synthesised based on the amino acid sequence of a larger snake venom toxin.

Preferred snakes include snakes from the family *Colubridae*, *Elapidae*, *Viperidae* and *Crotalidae* such as species of the genera *Naja*, *Dendroaspis*, *Bungarus*, *Pseudechis*, *Ophiophagus* and *Hemachatus*. Particularly preferred snakes are from the family *Elapidae* such as but not limited to King cobra (*Ophiophagus hannah*); True cobras (*Naja* spp); Asian or Indian cobra (*N. naja*); Egyptian cobra (*N. haje*); Spitting cobra (*N. nigricolli*); Black-lipped cobra (*N. malenoleuca*); Cape cobra (*N. nivea*); Gold's tree cobra (*Pseudohaje goldii*); Desert black snakes (*Walterinnesia* spp); Shield-nose snakes (*Aspidelaps* spp); Water cobras or water snakes (*Boulengerina* spp); Black mamba (*Dendroaspis polylepis*); Mamba (*D. angusticeps*); Kraits snake (*Bungarus* spp); Oriental coral snakes (*Calliophis* spp); Long-glanded coral snakes (*Maticora* spp); American coral snakes (*Micurus* spp); Southern coral snake (*M. frontalis*); Eastern coral snake or Harlequin snake (*M. fulvius*); Western coral snake (*Micruroides* spp); Arizona coral snake (*M. euryxanthus*); Death adder (*Acanthophis antarcticus*); Australian tiger snakes (*Notechis* spp); and Australian copperhead (*Denisomia* spp).

The present invention is exemplified with respect to peptides based on snake venom toxins from *O. hannah* and is particularly exemplified from *O. hannah* venom α -neurotoxins referred to as toxin a, toxin b and toxin (CM-11 (Joubert, 1973; Change *et al*, 1993; Song *et al*, 1994; Pu *et al*, 1995). However, the present invention extends to any toxin and more particularly α -neurotoxin from the venom of *O. hannah* as well as from the venom of any other snake such as from the family *Elapidae* or the families of *Colubridae*, *Viperidae* and *Crotalidae*.

According to a particularly preferred embodiment of the present invention, there is provided a peptide comprising a sequence of amino acids of from about 2 to about 50 residues having at least about 60% similarity to a sequence of amino acids from a α -

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neurotoxin isolatable from snake venom from a snake within the family *Elapidae* wherein said peptide is capable of inducing or facilitating analgesia in a mammal or a derivative, homologue, analogue or mimetic of said peptide.

More particularly, the present invention contemplates a peptide comprising from about
5 four to about 30 amino acid residues wherein all or a portion of the sequence has at least about 60% similarity to an α -neurotoxin from *O. hannah* snake venom and wherein said peptide induces or facilitates analgesia in a mammal.

The percentage similarity may be greater than 60% such as at least about 70% or at least about 8% or at least about 90% or higher.

10 Reference herein to a low stringency at 42°C includes and encompasses from at least about 1% v/v to at least about 15% v/v formamide and from at least about 1M to at least about 2M salt for hybridisation, and at least about 1M to at least about 2M salt for washing conditions. Alternative stringency conditions may be applied where necessary, such as
15 medium stringency, which includes and encompasses from at least about 16% v/v to at least about 30% v/v formamide and from at least about 0.5M to at least about 0.9M salt for hybridisation, and at least about 0.5M to at least about 0.9M salt for washing conditions, or high stringency, which includes and encompasses from at least about 31% v/v to at least about 50% v/v formamide and from at least about 0.01M to at least about 0.15M salt for hybridisation, and at least about 0.01M to at least about 0.15M salt for washing conditions.

20 Preferred α -neurotoxins from *O. hannah* include toxin a, toxin b and CM-11 amongst other α -neurotoxins such as those having an N-terminal amino acid sequence which includes the amino acid sequence C C S X_a X_b wherein

X_a is T or R;

X_b is D or T; and

25 X_c is N or K.

One particularly useful group of peptides of the present invention have analgesic properties inhibited by naloxone and N^ω-nitro-L-arginine methyl ester (L-NAME).

Although in no way intending to limit the present invention to any one theory or mode of action, this implies the involvement of the opioid and nitric oxide systems, respectively.

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Accordingly, another preferred embodiment of the present invention provides a peptide having the following characteristics:

- (i) induces or facilitates analgesia in a mammal;
- (ii) comprises from about 2 to about 50 amino acid residues;
- 5 (iii) its analgesic ability is capable of inhibition by naloxone and or L-NAME; and
- (iv) comprises an amino acid sequence of which at least four amino acid residues are common to the amino acid sequence of an α -neurotoxin from *O. hannah*;

or a derivative, homologue, analogue or mimetic of said peptide.

A particularly preferred peptide of the present invention comprises the amino acid
 10 sequence N P F P T [SEQ ID NO:1] and even more preferably the amino acid sequence
 N P F P T X₁ X₂ K R X₃ [SEQ ID NO:2] wherein X₁, X₂, X₃ and X₄ may be the same or
 different and each is an amino acid residue. Preferably, X₁ is absent or is W, X₂ is
 absent or is R, X₃ is P or K and X₄ is absent or is G or H. Most preferably, the peptide
 comprises the amino acid sequences:

- 15 N P F P T W R K R P G [SEQ ID NO:3];
- N P F P T R K R P [SEQ ID NO:4];
- N P F P T W R K R P [SEQ ID NO:5]; and
- N P F P T W K R K H [SEQ ID NO:6];

or derivatives, homologues, analogues or mimetics thereof.

- 20 Reference herein to "derivatives" includes parts, fragments and portions of the subject
 analgesic peptides. A derivative also includes a single or multiple amino acid
 substitution, deletion and/or addition. Homologues include functionally, structurally or
 stereochemically similar peptides from venom from the same species of snake or from
 within the same genus or family of snake. For example, a homologue would include a
 25 peptide having, for example, the amino acid sequence N P F P from another α -
 neurotoxin from another member of the *Elapidae* family. Alternatively, the amino acids
 N P F P may be replaced by amino acids having a similar charge, stereochemistry,
 conformation or peptide conformation influencing ability. All such homologues are
 contemplated by the present invention.

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Analogues and mimetics include molecules which include molecules which contain non-naturally occurring amino acids or which do not contain amino acids but nevertheless behave functionally the same as the peptide. Natural product screening is one useful strategy for identifying analogues and mimetics.

- 5 Although, analogues of the subject peptides contemplated herein include modifications to side chains, incorporation of unnatural amino acids and/or their derivatives during peptide synthesis and the use of crosslinkers and other methods which impose conformational constraints on the peptide molecule or their analogues.

- Examples of side chain modifications contemplated by the present invention include
- 10 modifications of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with NaBH_4 ; amidination with methylacetimidate; acylation with acetic anhydride; carbamoylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS); acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and
- 15 pyridoxylation of lysine with pyridoxal-5-phosphate followed by reduction with NaBH_4 .

The guanidine group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

The carboxyl group may be modified by carbodiimide activation *via* O-acylisourea formation followed by subsequent derivitisation, for example, to a corresponding amide.

- 20 Sulphydryl groups may be modified by methods such as carboxymethylation with iodoacetic acid or iodoacetamide; performic acid oxidation to cysteic acid; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate, 4-chloromercuriphenylsulphonic acid, phenylmercury chloride, 2-chloromercuri-4-nitrophenol and other mercurials; carbamoylation with cyanate at alkaline
- 25 pH.

Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide

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or sulphenyl halides. Tyrosine residues on the other hand, may be altered by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carbethoxylation with
5 diethylpyrocarbonate.

Examples of incorporating unnatural amino acids and derivatives during peptide synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid, t-butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid, 2-thienyl
10 alanine and/or D-isomers of amino acids. A list of unnatural amino acid contemplated herein is shown in Table 2.

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TABLE 2

	Non-conventional amino acid	Code	Non-conventional amino acid	Code
5				
	α -aminobutyric acid	Abu	L-N-methylalanine	Nmala
	α -amino- α -methylbutyrate	Mgab	L-N-methylarginine	Nmarg
	aminocyclopropane- carboxylate	Cpro	L-N-methylasparagine	Nmasn
			L-N-methylaspartic acid	Nmasp
10	aminoisobutyric acid	Aib	L-N-methylcysteine	Nmcys
	aminonorbornyl- carboxylate	Norb	L-N-methylglutamine	Nmgln
			L-N-methylglutamic acid	Nmglu
	cyclohexylalanine		Chexa L-N-methylhistidine	Nmhis
	cyclopentylalanine	Cpen	L-N-methylisoleucine	Nmile
15	D-alanine	Dal	L-N-methylleucine	Nmleu
	D-arginine	Darg	L-N-methyllysine	Nmlys
	D-aspartic acid	Das	L-N-methylmethionine	Nmmet
	D-cysteine	Dcys	L-N-methylnorleucine	Nmnle
	D-glutamine	Dgln	L-N-methylnorvaline	Nmnva
20	D-glutamic acid	Dglu	L-N-methylornithine	Nmorn
	D-histidine	Dhis	L-N-methylphenylalanine	Nmphe
	D-isoleucine	Dile	L-N-methylproline	Nmpro
	D-leucine	Dleu	L-N-methylserine	Nmser
	D-lysine	Dlys	L-N-methylthreonine	Nmthr
25	D-methionine	Dmet	L-N-methyltryptophan	Nmtrp
	D-ornithine	Dom	L-N-methyltyrosine	Nmtyr
	D-phenylalanine	Dphe	L-N-methylvaline	Nmval
	D-proline	Dpro	L-N-methylethylglycine	Nmetg
	D-serine	Dser	L-N-methyl-t-butylglycine	Nmtbug
30	D-threonine	Dthr	L-norleucine	Nle
	D-tryptophan	Dtrp	L-norvaline	Nva

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	D-tyrosine	Dtyr	α -methyl-aminoisobutyrate	Maib
	D-valine	Dval	α -methyl- γ -aminobutyrate	Mgabv
	D- α -methylalanine	Dmala	α -methylcyclohexylalanine	Mchexa
	D- α -methylarginine	Dmarg	α -methylcyclopentylalanine	Mcpen
5	D- α -methylasparagine	Dmasn	α -methyl- α -naphthylalanine	Manap
	D- α -methylaspartate	Dmasp	α -methylpenicillamine	Mpen
	D- α -methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
	D- α -methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
	D- α -methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norn
10	D- α -methylisoleucine	Dmile	N-amino- α -methylbutyrate	Nmaabu
	D- α -methylleucine	Dmleu	α -naphthylalanine	Anap
	D- α -methyllysine	Dmlys	N-benzylglycine	Nphe
	D- α -methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
	D- α -methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
15	D- α -methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
	D- α -methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
	D- α -methylserine	Dmse	N-cyclobutylglycine	Ncbut
	D- α -methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
	D- α -methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
20	D- α -methyltyrosine	Dmty	N-cyclodecylglycine	Ncdec
	D- α -methylvaline	Dmval	N-cyclododecylglycine	Ncdod
	D-N-methylalanine	Dnmala	N-cyclooctylglycine	Ncoct
	D-N-methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
	D-N-methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
25	D-N-methylaspartate	Dnmasp	N-(2,2-diphenylethyl)glycine	Nbhm
	D-N-methylcysteine	Dnmcys	N-(3,3-diphenylpropyl)glycine	Nbhe
	D-N-methylglutamine	Dnmgln	N-(3-guanidinopropyl)glycine	Narg
	D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
	D-N-methylhistidine	Dnmhis	N-(hydroxyethyl)glycine	Nser
30	D-N-methylisoleucine	Dnmile	N-(imidazolylethyl)glycine	Nhis
	D-N-methylleucine	Dnmleu	N-(3-indolylethyl)glycine	Nhtrp

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	D-N-methyllysine	Dnmlys	N-methyl- γ -aminobutyrate	Nmgabu
	N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmt
	D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpn
	N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
5	N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
	N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
	N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
	D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
	D-N-methyltyrosine	Dnmtyr	N-methyl- α -naphthylalanine	Nmanap
10	D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
	γ -aminobutyric acid	Gabu	N-(<i>p</i> -hydroxyphenyl)glycine	Nhtyr
	L- <i>t</i> -butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
	L-ethylglycine	Etg	penicillamine	Pen
	L-homophenylalanine	Hphe	L- α -methylalanine	Mala
15	L- α -methylarginine	Marg	L- α -methylasparagine	Masn
	L- α -methylaspartate	Masp	L- α -methyl- <i>t</i> -butylglycine	Mtbug
	L- α -methylcysteine	Mcys	L-methylethylglycine	Metg
	L- α -methylglutamine	Mgln	L- α -methylglutamate	Mglu
	L- α -methylhistidine	Mhis	L- α -methylhomophenylalanine	Mhphe
20	L- α -methylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
	L- α -methylleucine	Mleu	L- α -methyllysine	Mlys
	L- α -methylmethionine	Mmet	L- α -methylnorleucine	Mnle
	L- α -methylnorvaline	Mnva	L- α -methylornithine	Morn
	L- α -methylphenylalanine	Mphe	L- α -methylproline	Mpro
25	L- α -methylserine	Mser	L- α -methylthreonine	Mthr
	L- α -methyltryptophan	Mtrp	L- α -methyltyrosine	Mtyr
	L- α -methylvaline	Mval	L-N-methylhomophenylalanine	Nmhphe
	N-(N-(2,2-diphenylethyl)	Nnbhm	N-(N-(3,3-diphenylpropyl)	Nnbhe
	carbamylmethyl)glycine		carbamylmethyl)glycine	
30	1-carboxy-1-(2,2-diphenyl-	Nmbc		
	ethylamino)cyclopropane			

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Crosslinkers can be used, for example, to stabilise 3D conformations, using homo-bifunctional crosslinkers such as the bifunctional imido esters having $(CH_2)_n$ spacer groups with $n=1$ to $n=6$, glutaraldehyde, N-hydroxysuccinimide esters and hetero-bifunctional reagents which usually contain an amino-reactive moiety such as N-hydroxysuccinimide and another group specific-reactive moiety such as maleimido or dithio moiety (SH) or carbodiimide (COOH). In addition, peptides can be conformationally constrained by, for example, incorporation of C_α and N_α -methylamino acids, introduction of double bonds between C_α and C_β atoms of amino acids and the formation of cyclic peptides or analogues by introducing covalent bonds such as forming an amide bond between the N and C termini, between two side chains or between a side chain and the N or C terminus.

The present invention further contemplates chemical analogues of the subject peptides capable of acting as antagonists or agonists of the analgesic peptides. Chemical analogues may not necessarily be derived from the peptides themselves but may share certain conformational similarities. Alternatively, chemical analogues may be specifically designed to mimic certain physiochemical properties of the peptides. Chemical analogues may be chemically synthesised or may be detected following, for example, natural product screening.

All these types of modifications may be important to stabilise the subject peptide if administered to a subject.

As stated above, the peptides of the present invention are useful in inducing or facilitating analgesia in a subject such as a mammal (eg. a human).

Accordingly, another aspect of the present invention contemplates a method of inducing analgesia in a subject said method comprising administering to said subject a pain alleviating effective amount of a peptide as hereinbefore defined for a time and under conditions sufficient to induce analgesia or to otherwise reduce the level of pain.

Preferably, the peptide administered comprises an amino acid sequence having at least about 60% similarity to an α -neurotoxin from *O. hannah* or is a derivative, homologue,

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analogue or mimetic thereof.

In accordance with this method, more than one type of peptide may be administered or the peptide may be co-administered with a known analgesic compound or molecule. By "co-administered" is meant simultaneous administration in the same formulation or in two
5 different formulations *via* the same or different routes or sequential administration by the same or different routes. By "sequential" administration is meant a time difference of from seconds, minutes, hours or days between the administration of the two types of peptides or the peptide and the known analgesic compound or molecule. The peptides or the peptide and known analgesic compound or molecule may be administered in any
10 order.

Routes of administration include but are not limited to intravenously, intraperitoneal, subcutaneously, intracranial, intradermal, intramuscular, intraocular, intrathecal, intracerebrally, intranasally, infusion, orally, rectally, *via* iv drip, patch and implant. Intravenous and intraperitoneal and anal routes are particularly preferred.

- 15 Another aspect of the present invention provides a composition for use inducing or facilitating analgesia comprising a peptide as hereinbefore defined and one or more pharmaceutically acceptable carriers and/or diluents. The composition may also comprise two different types of peptides or a peptide and a known analgesic compound or molecule.
- 20 Compositions suitable for injectable use include sterile aqueous solutions (where water soluble) and sterile powders for the extemporaneous preparation of sterile injectable solutions. They must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water,
25 ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof and vegetable oils. The preventions of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium

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chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

5 Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by, for example, filter sterilization or sterilization by other appropriate means. Dispersions are also contemplated and these may be prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those
10 enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, a preferred method of preparation includes vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution.

When the active ingredients are suitably protected, they may be orally administered, for
15 example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by
20 weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in such therapeutically useful compositions in such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains
25 between about 0.1mg and 2000 mg of active compound.

The tablets, troches, pills, capsules and the like may also contain the components as listed hereafter: A binder such as gum, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such a
30 sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of

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wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or
5 elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound(s) may be incorporated into sustained-release preparations and formulations.

- 10 The present invention also extends to forms suitable for topical application such as creams, lotions and gels. In such forms, the analgesic peptides may need to be modified to permit penetration of the surface barrier.

Pharmaceutically acceptable carriers and/or diluents include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption
15 delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

- 20 It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required pharmaceutical
25 carrier. The specification for the novel dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved and (b) the limitations inherent in the art of compounding such an active material for induce or facilitating analgesia in living subjects.

Effective amounts of analgesic peptides contemplated by the present invention will vary

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depending on the severity of the pain and the health and age of the recipient. In general terms, effective amounts may vary from 0.01 ng/kg body weight to about 100 mg/kg body weight. Alternative amounts include for about 0.1 ng/kg body weight about 100 mg/kg body weight or from 1.0 ng/kg body weight to about 80 mg/kg body weight.

- 5 Still another aspect of the present invention is directed to antibodies to the subject peptides and their derivatives, homologues, analogues and mimetics. Such antibodies may be monoclonal or polyclonal.

In the case of small peptides, these may first need to be associated with a carrier molecule.

- The antibodies of the present invention are particularly useful as therapeutic or diagnostic
10 agents. For example, specific antibodies can be used to screen for peptides using immunoassays or used as antagonists to inhibit peptide activity under certain circumstances such as where temporary analgesia is only required. Techniques for such immunoassays are well known in the art and include, for example, sandwich assays and ELIZA. Knowledge of peptide levels may be important for monitoring certain therapeutic
15 protocols.

- Antibodies to the peptides (or their derivatives, homologues, analogues or mimetics) of the present invention may be monoclonal or polyclonal. Alternatively, fragments of antibodies may be used such as Fab fragments. Furthermore, the present invention extends to recombinant and synthetic antibodies and to antibody hybrids. A "synthetic antibody" is
20 considered herein to include fragments and hybrids of antibodies.

As stated above, specific antibodies can be used to screen for the subject peptides. The latter would be important, for example, as a means for screening for levels of peptides in a cell extract or other biological fluid or purifying peptides made by recombinant means from culture supernatant fluid.

- 25 It is within the scope of this invention to include any second antibodies (monoclonal, polyclonal or fragments of antibodies or synthetic antibodies) directed to the first mentioned antibodies discussed above. Both the first and second antibodies may be used in detection assays or a first antibody may be used with a commercially available anti-

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immunoglobulin antibody. An antibody as contemplated herein includes any antibody specific to any region of peptide.

Both polyclonal and monoclonal antibodies are obtainable by immunization with the enzyme or protein and either type is utilizable for immunoassays. The methods of obtaining both types of sera are well known in the art. Polyclonal sera are less preferred but are relatively easily prepared by injection of a suitable laboratory animal with an effective amount of peptide, or antigenic parts thereof, collecting serum from the animal and isolating specific sera by any of the known immunoadsorbent techniques. Although antibodies produced by this method are utilizable in virtually any type of immunoassay, they are generally less favoured because of the potential heterogeneity of the product.

The use of monoclonal antibodies in an immunoassay is particularly preferred because of the ability to produce them in large quantities and the homogeneity of the product. The preparation of hybridoma cell lines for monoclonal antibody production derived by fusing an immortal cell line and lymphocytes sensitized against the immunogenic preparation can be done by techniques which are well known to those who are skilled in the art.

Another aspect of the present invention contemplates a method for detecting a subject peptide in a biological sample from a subject or culture supernatant flow or other source said method comprising contacting said biological sample with an antibody specific for said peptide or its derivative, homologue, analogue or mimetic for a time and under conditions sufficient for an antibody-peptide complex to form, and then detecting said complex.

The presence of a peptide may be accomplished in a number of ways such as by Western blotting and ELISA procedures. A wide range of immunoassay techniques are available as can be seen by reference to US Patent Nos. 4,016,043, 4,424,279 and 4,018,653. These, of course, include both single-site and two-site or "sandwich" assays of the non-competitive types, as well as in the traditional competitive binding assays. These assays also include direct binding of a labelled antibody to a target.

Sandwich assays are among the most useful and commonly used assays and are favoured for use in the present invention. A number of variations of the sandwich assay technique

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exist, and all are intended to be encompassed by the present invention. Briefly, in a typical forward assay, an unlabelled antibody is immobilized on a solid substrate and the sample to be tested brought into contact with the bound molecule. After a suitable period of incubation, for a period of time sufficient to allow formation of an antibody-antigen complex, a second antibody specific to the antigen, labelled with a reporter molecule 5 capable of producing a detectable signal is then added and incubated, allowing time sufficient for the formation of another complex of antibody-antigen-labelled antibody. Any unreacted material is washed away, and the presence of the antigen is determined by observation of a signal produced by the reporter molecule. The results may either be 10 qualitative, by simple observation of the visible signal, or may be quantitated by comparing with a control sample containing known amounts of hapten. Variations on the forward assay include a simultaneous assay, in which both sample and labelled antibody are added simultaneously to the bound antibody. These techniques are well known to those skilled in the art, including any minor variations as will be readily apparent. In accordance with the 15 present invention the sample is one which might contain an analgesic peptide including cell extract, culture supernatant tissue biopsy, serum, saliva, mucosal secretions, lymph, tissue fluid and respiratory fluid. The sample is, therefore, generally a biological sample comprising biological fluid but also extends to fermentation fluid and supernatant fluid such as from a cell culture.

20 In the typical forward sandwich assay, a first antibody having specificity for the peptide or antigenic parts thereof is either covalently or passively bound to a solid surface. The solid surface is typically glass or a polymer, the most commonly used polymers being cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene. The solid supports may be in the form of tubes, beads, discs of microplates, or any other surface 25 suitable for conducting an immunoassay. The binding processes are well-known in the art and generally consist of cross-linking covalently binding or physically adsorbing, the polymer-antibody complex is washed in preparation for the test sample. An aliquot of the sample to be tested is then added to the solid phase complex and incubated for a period of time sufficient (e.g. 2-40 minutes or overnight if more convenient) and under suitable 30 conditions (e.g. from room temperature to about 37°C) to allow binding of any subunit present in the antibody. Following the incubation period, the antibody subunit solid phase

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is washed and dried and incubated with a second antibody specific for a portion of the hapten. The second antibody is linked to a reporter molecule which is used to indicate the binding of the second antibody to the hapten.

An alternative method involves immobilizing the target molecules in the biological sample
5 and then exposing the immobilized target to specific antibody which may or may not be labelled with a reporter molecule. Depending on the amount of target and the strength of the reporter molecule signal, a bound target may be detectable by direct labelling with the antibody. Alternatively, a second labelled antibody, specific to the first antibody is exposed to the target-first antibody complex to form a target-first antibody-second
10 antibody tertiary complex. The complex is detected by the signal emitted by the reporter molecule.

By "reporter molecule" as used in the present specification, is meant a molecule which, by its chemical nature, provides an analytically identifiable signal which allows the detection of antigen-bound antibody. Detection may be either qualitative or quantitative. The most
15 commonly used reporter molecules in this type of assay are either enzymes, fluorophores or radionuclide containing molecules (i.e. radioisotopes) and chemiluminescent molecules.

In the case of an enzyme immunoassay, an enzyme is conjugated to the second antibody, generally by means of glutaraldehyde or periodate. As will be readily recognized, however, a wide variety of different conjugation techniques exist, which are readily
20 available to the skilled artisan. Commonly used enzymes include horseradish peroxidase, luciferase glucose oxidase, beta-galactosidase and alkaline phosphatase, amongst others. The substrates to be used with the specific enzymes are generally chosen for the production, upon hydrolysis by the corresponding enzyme, of a detectable colour change. Examples of suitable enzymes include alkaline phosphatase and peroxidase. It is also
25 possible to employ fluorogenic substrates which yield a fluorescent product rather than the chromogenic substrates noted above. In all cases, the enzyme-labelled antibody is added to the first antibody-peptide complex, allowed to bind, and then the excess reagent is washed away. A solution containing the appropriate substrate is then added to the complex of antibody-antigen-antibody. The substrate will react with the enzyme linked to the second
30 antibody, giving a qualitative visual signal, which may be further quantitated, usually

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spectrophotometrically, to give an indication of the amount of hapten which was present in the sample. "Reporter molecule" also extends to use of cell agglutination or inhibition of agglutination such as red blood cells on latex beads, and the like.

Alternately, fluorescent compounds, such as fluorescein and rhodamine, may be
5 chemically coupled to antibodies without altering their binding capacity. When activated by illumination with light of a particular wavelength, the fluorochrome-labelled antibody adsorbs the light energy, inducing a state to excitability in the molecule, followed by emission of the light at a characteristic colour visually detectable with a light microscope. As in the EIA, the fluorescent labelled antibody is allowed to bind to the first antibody-
10 hapten complex. After washing off the unbound reagent, the remaining tertiary complex is then exposed to the light of the appropriate wavelength the fluorescence observed indicates the presence of the hapten of interest. Immunofluorescence and EIA techniques are both very well established in the art and are particularly preferred for the present method. However, other reporter molecules, such as radioisotope, chemiluminescent or
15 bioluminescent molecules, may also be employed.

The present invention further contemplates recombinant analgesic peptides. Accordingly, another aspect of the present invention provides a nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a peptide as hereinbefore defined or a derivative or homologue thereof.

20 A particularly useful nucleic acid molecule is one which encodes or is complementary to a sequence which encodes a peptide amino acid sequence N P F P R [SEQ ID NO:1], said peptide capable of inducing or facilitating analgesia or a derivative or homologue of said peptide.

Even more preferably, the nucleic acid comprises a nucleotide sequence encoding or
25 complementary to a sequence encoding the amino acid sequence

N P F P T X₁ X₂ K R X₃ X₄ [SEQ ID NO:2]

where X₁, X₂, X₃ and X₄ may be the same or different and each is any amino acid residue, said peptide capable of inducing or facilitating analgesia or a derivative or

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homologue of said peptide.

Still yet more particularly, the nucleic acid molecule comprises a sequence of nucleotides encoding or complementary to a sequence encoding an amino acid sequence selected from:

- 5 N P R P T W R K R P [SEQ ID NO:1];
N P F P T R K R P [SEQ ID NO:2];
N P F P T W R K R P [SEQ ID NO:3]; and
N P F P R W K R K H [SEQ ID NO:4].

The nucleic acid molecule of the present invention is generally in isolated form. It may
10 also comprise additional nucleotide sequence information fused, linked or otherwise
~~associated with it either at the 3' or 5' terminal portions or at both the 3' and 5' terminal~~
portions. The nucleic acid molecule may also be part of a vector, such as an expression
vector. The latter embodiment facilitates production of recombinant forms of the subject
peptides which forms are encompassed by the present invention.

15 The present invention further encompasses host cells for the subject nucleic acid
molecules and which are used to produce recombinant peptides. The host cells may be
prokaryotic cells or eukaryotic cells. Examples of prokaryotic cells include *E. coli*,
Bacillus sp, *Pseudomonas* sp amongst many others. Examples of eukaryotic cells
include mammalian cell lines (eg. CHO cells), yeast cells, fungal cells, insect cells, plant
20 cells and reptilian cell lines. The ability to produce recombinant peptides of the present
invention permits the large scale production of vast quantities of peptides for commercial
uses. As stated above, the peptide may need to be produced as part of a large peptide,
polypeptide or protein which may be used as is or may first need to be processed in order
to remove the extraneous proteinaceous sequences. Such processing includes digestion
25 with proteases, peptidases and amidases or a range of chemical, electrochemical, sonic or
mechanical disruption techniques.

Notwithstanding that the present invention encompasses recombinant peptides,
chemically synthetic techniques are particularly preferred in the synthesis of the peptides.

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The peptide fragments such as those set forth in SEQ ID NO:3 to 6 are conveniently synthesized based on larger toxin molecules isolated from snake venom. Isolation of the larger toxin molecules may be accomplished by any suitable means such as by chromatographic separation, for example using CM-cellulose ion exchange chromatography followed by Sephadex (eg. G-50 column) filtration. Many other techniques are available including HPLC, PAGE amongst others. Once purified, the toxin can be partially sequenced and/or fragments produced and used directly as a source of peptides or as a template for peptide synthesis.

Peptides may be synthesized by solid phase synthesis using F-moc chemistry as described by Carpino *et al* (1991). The peptide and fragments thereof may also be synthesized by alternative chemistries including, but not limited to, t-Boc chemistry as described in Stewwart *et al* (1985) or by either classical methods of liquid phase peptide synthesis. The synthesized peptides are conveniently tested for analgesic activity in a hot-plate test (see Examples).

The present invention further extends to the use of the subject peptides in the manufacture of a medicament for inducing or facilitating analgesia.

Preferred peptides are those which comprises the amino acid sequence N P F P T [SEQ ID NO:1], and more preferably the amino acid sequence

N P F P T X₁ X₂ X₃ X₄ [SEQ ID NO:2]

wherein X₁, X₂, X₃, X₄ may be the same or different and each is any amino acid.

Particularly preferred peptides are selected from:

N P F P T W R K R P G [SEQ ID NO:3];

N P F P T R K R P [SEQ ID NO:4];

N P F P T W R K R P [SEQ ID NO:5]; and

N P F P T W K R K H [SEQ ID NO:6].

Yet another aspect of the present invention provides an agent useful or inducing or facilitating analgesia comprising a peptide as hereinbefore defined.

The present invention is further described by the following non-limiting Examples.

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EXAMPLE 1**Animals**

Swiss albino mice (female, 18-22 g) were obtained from the University Laboratory Animal Centre, National University of Singapore, Singapore.

5

EXAMPLE 2**Peptide Synthesis**

The peptide fragments

H₂N - N P F P T W R K R P G - COOH [SEQ ID NO:1]

H₂N - N P F P T R K R P - COOH [SEQ ID NO:2];

10 H₂N - N P F P T W R K R P - COOH [SEQ ID NO:3]; and

H₂N - N P F P T W K R K H - COOH [SEQ ID NO:4];

were synthesized based on the 72 amino acid sequence of hannahalgesin (Pu *et al*, 1995) and the corresponding homologous sequences in toxin a and toxin b (Joubert, 1973).

15 The first 10 amino acids of peptide -N-P-F-P-T-W-R-K-R-P is homologous or shows a high degree of homology with the terminal portions of the King cobra toxins; the last amino acid, glycine, has no significance as it was part of the synthetic protein fragments used to synthesize the peptide. The peptide was synthesized by solid phase synthesis using F-moc chemistry as described by Carpino. Table 3 provides a comparison of amino acid sequences from the peptide fragment.

20 The synthesized peptide was tested for analgesic activity in the hot-plate test. The neurotoxic effects was also tested on the chick biventral.

EXAMPLE 3**Chick Biventral Experiment**

Local domestic chicks (*Gallus domesticus*) of 5 to ten days old were killed with CO₂; the

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upper oesophagus up to the crop was removed and mounted under 1 g tension in 6 ml Krebs solution, maintained at 32°C and aerated with 5% v/v CO₂ in oxygen.

Electrical field stimulation (EFS:25V, 5Hz x 10 S, 1 MS pulse width, every 100s) was applied to produce contractile responses of the oesophagi. Responses were recorded on a Grass polygraph via a force displacement transducer (model FT03, Grass). After the contraction of oesophagi reached stability, the synthesized peptide (10µG) was added to see whether it blocked the contractile response.

As shown in Figure 1, after adding an analgesic peptide, the contractile response of chick biventer showed no change. Therefore, the peptide had no neurotoxic effect on the neuromuscular junction.

EXAMPLE 4

Hot-plate test

Mice were placed on a hot plate (55°C±0.5°C) and confined in an area of 4 x 6 inches by a 5-inch tall transparent plastic wall. The latency time was measured from the time the mouse was put on the hot plate to the time when it first jumps or licks its hindlimb. The hot-plate test was carried out before and after drug administration by i.v. (in vein, 72 ng/g) or p.o. (per oral, 360ng/g).

Compared to the saline-administered controls, the peptide administered i.v. (72ng/g) or p.o. (360ng/g) produced significant increases in hot-plate latency time (Figure 2). The latency times obtained before and after i.v. or p.o. administration were significantly different. The synthesized peptide, therefore, has analgesic activity.

EXAMPLE 5

Effects of naloxone and L-NAME

Mice were treated with the peptide i.v. at doses of 72ng/g. After 1.5 h, the mice were injected i.v. with naloxone (6mg/kg) and the hot-plate tests were performed another 30

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minutes later.

An inhibitor of NO synthase, L-N^G-nitro arginine methyl ester (L-NAME), was used to determine whether NO was involved in the hannahalgesin induced antinociception. Mice were treated i.v. with the peptide (72ng/g), and 1.5 hours later, injected i.v. with L-NAME (20mg/kg). The hot-plate tests were performed another 30 minutes later.

Naloxone effectively attenuated the analgesic effects of the peptide as shown in Figure 3.

Similarly, the coadministration of L-NAME with the peptide also significantly decreased the analgesic effect induced by the peptide (Figure 3).

As both naloxone and L-NAME blocked the analgesic effect of this peptide, the involvement of the opioid and nitric oxide systems is suggested.

TABLE 3

Homology of Similar Peptide Segments

Syn. Peptide	N P	F P T W R K	R P (G)
CM-11	N P	F P T R P	R P
Toxin - a	N P	F P T W R K	R P
Toxin -b	NP	F P T W K	R K (H)

*Excluding amino acids, in parentheses

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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BIBLIOGRAPHY

1. Tin, M., Rai, M, Maung, C., Tun, P. and Da, W (1991) Bites by the King cobra (*Ophiophagus hannah*) in Myanmar: successful treatment of severe neurotoxic envenoming. *Q. J. Med.* 80, 751-762.
- 5 2. Joubert, F.J. (1973) The amino acid sequences of two toxins from *Ophiophagus hannah* (king cobra) venom. *Biochim. biophys. Acta* 317, 85-98.
3. Chang, C. C., Huang, T.Y., Kuo, K.W., Chen, S.W., Huang, K.F. and Chiou, S. H. (1993) Sequence characterisation of a novel α -neurotoxin from the king cobra (*Ophiophagus hannah*) venom. *Biochem, biophys. Res. Commun.* 191, 214-223.
- 10 4. Chen, R. and Robinson, S.E. (1990) The effect of cholinergic manipulations on the analgesic response to cobrotoxin in mice. *Life Sci.* 21, 1949-1954.
5. Song, J., Chung, M. C. M., Xiong, Y., Wang, W. and Pu, X. (1994) Purification, sequence and pharmacological studies of a new alpha-neurotoxin from *Ophiophagus hannah* venom. *Toxicon* 32, 537-538 (Abstract).
- 15 6. X. C. Pu, P. T. H. Wong and P Gopalakrishnakone. (1995) A novel analgesic toxin (Hannalgesin) from the venom of king cobra (*Ophiophagus hannah*). *Toxicon* 33, No 11, 1425-1431.
7. Carpino, L.A., Chao, H.G., Beyermann, M. and Biernert, M. (1991) [(9-Fluorenylmethyl)oxy]carbonyl amino acid chlorides in solid-phase peptide synthesis. *Journal of Organic Chemistry*, 56, 2635-2642.
- 20 8. Stewaart, J. M. and Young, J. D. Eds (1985) *Solid Phase Peptide Synthesis*, 2nd Edition, pp 1-157, Pierce Chemical, Rockford.

CLAIMS

1. An isolated peptide comprising a sequence of amino acids having at least 60% similarity to a sequence of amino acids from a portion of a toxin from snake venom and which peptide is capable of inducing or facilitating analgesia in a host or a derivative, homologue, analogue or mimetic of said peptide.
2. An isolated peptide according to claim 1 wherein the snake venom is from a snake from the family *Elapidae*, *Viperidae*, *Colubridae* or *Crotalidae*.
3. An isolated peptide according to claim 2 wherein the snake venom is from a snake from the family *Elapidae*.
4. An isolated peptide according to claim 3 wherein the snake venom is from a snake selected from *Ophiophagus hannah*, *Naja* spp, *Naja naja*, *Naja haje*, *Naja nigricolli*, *Naja melanoleuca*, *Naja nivea*, *Pseudohaje goldii*, *Walterinnesia* spp, *Aspidelaps* spp, *Boulengerina* spp, *Dendroaspis polylepis*, *Bungarus* spp, *Notechis* spp, *Denisonia* spp and *Micurus* spp.
5. An isolated peptide according to claim 4 wherein the snake venom is from *O. hannah*.
6. An isolated peptide according to claim 5 comprising the amino acid sequence N P F T [SEQ ID NO:1].
7. An isolated peptide according to claim 6 comprising the amino acid sequence
N P F P T X₁ X₂ K R X₃ X₄ [SEQ ID NO:2]
wherein X₁, X₂, X₃ and X₄ may be the same or different and each is any amino acid residue.
8. An isolated peptide according to claim 7 wherein
X₁ is absent or is W;
X₂ is absent or is R;
X₃ is P or K; and
X₄ is absent or is G or H.

9. An isolated peptide according to claim 8 comprising the amino acid sequence
N P F P T W R K R P G [SEQ ID NO:3].
10. An isolated peptide according to claim 8 comprising the amino acid sequence
N P F P T R K R P [SEQ ID NO:4].
- 5 11. An isolated peptide according to claim 8 comprising the amino acid sequence
N P F P T W R K R P [SEQ ID NO:5].
12. An isolated peptide according to claim 8 comprising the amino acid sequence
N P F P T W K R K H [SEQ ID NO:6].
13. An isolated peptide according to claim 8 wherein the analgesic properties of the
10 peptide are inhibited by naloxone and/or L-NAME.
14. A method of including analgesia in a subject said method comprising
administering to said subject a pain alleviating effective amount of a peptide which
comprises a sequence of amino acids from a portion of a toxin from snake venom.
15. A method according to claim 14 wherein the snake venom is from a snake from
15 the family *Elapidae*, *Viperidae*, *Colubridae* or *Crotalidae*.
16. A method according to claim 15 wherein the snake venom is from a snake from
the family *Elapidae*.
17. A method according to claim 16 wherein the snake venom is from a snake
selected from *Ophiophagus hannah*, *Naja* spp, *Naja naja*, *Naja haje*, *Naja nigricolli*,
20 *Naja melanoleuca*, *Naja nivea*, *Pseudohaje goldii*, *Walterinnesia* spp, *Aspidelaps* spp,
Boulengerina spp, *Dendroaspis polylepis*, *Bungarus* spp, *Notechis* spp, *Denisonia* spp
and *Micurus* spp.
18. A method according to claim 17 wherein the snake venom is from *O. hannah*.
19. A method according to claim 18 wherein the peptides comprises the amino acid
25 sequence N P F P T [SEQ ID NO:1].

20. A method according to claim 19 wherein the peptide comprises the amino acid sequence

N P F P T X₁ X₂ K R X₃ X₄ [SEQ ID NO:2]

wherein X₁, X₂, X₃ and X₄ may be the same or different and each is any amino acid
5 residue.

21. A method according to claim 20 wherein

X₁ is absent or is W;

X₂ is absent or is R;

X₃ is P or K; and

10 X₄ is absent or is G or H.

22. A method according to claim 21 wherein the peptide comprises the amino acid sequence

N P F P T W R K R P G [SEQ ID NO:3].

23. A method according to claim 21 wherein the peptide comprises the amino acid
15 sequence

N P F P T R K R P [SEQ ID NO:4].

24. A method according to claim 21 wherein the peptide comprises the amino acid sequence

N P F P T W R K R P [SEQ ID NO:5].

20 25. A method according to claim 21 wherein the peptide comprises the amino acid sequence

N P F P T W K R K H [SEQ ID NO:6].

26. A method according to claim 21 wherein the analgesic properties of the peptide are inhibited by naloxone and/or L-NAME.

25 27. An antibody specific for the peptide according to any one of claims 7 or 8 or 9 or 10 or 11 or 12.

28. An antibody according to claim 27 wherein the antibody is a monoclonal

antibody.

29. A composition useful of inducing pain relief, said composition comprising a peptide comprising a sequence of amino acids having at least 60% similarity to a sequence of amino acids from a portion of a toxin from snake venom, said composition
5 further comprising one or more pharmaceutically acceptable carriers and/or diluents.

30. A composition according to claim 29 wherein the snake venom is from a snake from the family *Elapidae*, *Viperidae*, *Colubridae* or *Crotalidae*.

31. A composition according to claim 30 wherein the snake venom is from a snake from the family *Elapidae*.

10 32. A composition according to claim 31 wherein the snake venom is from a snake selected from *Ophiophagus hannah*, *Naja* spp, *Naja naja*, *Naja haje*, *Naja nigricolli*, *Naga melanoleuca*, a snake from the family *Elapidae*, *Viperidae*, *Colubridae* or *Crotalidae*.

33. A composition according to claim 32 wherein the snake venom is from a snake
15 from the family *Elapidae*.

34. A composition according to claim 33 wherein the snake venom is from a snake selected from *Ophiophagus hannah*, *Naja* spp, *Naja naja*, *Naja haje*, *Naja nigricolli*, *Naga melanoleuca*, *Naja nivea*, *Pseudohaje goldii*, *Walterinesia* spp, *Aspidelaps* spp, *Boulengerina* spp, *Dendroaspis polylepis*, *Bungarus* spp, *Notechis* sp, *Denisonia* spp and
20 *Micurus* spp.

35. A composition according to claim 34 wherein the snake is from *O. hannah*.

36. A composition according to claim 35 wherein the peptide comprises the amino acid sequence N P F P T [SEQ ID NO:1].

37. A composition according to claim 36 wherein the peptide comprises the amino
25 acid sequence

N P F P T X₁ X₂ K R X₃ X₄ [SEQ ID NO:2]

wherein X_1 , X_2 , X_3 and X_4 may be the same or different and each is any amino acid residue.

38. A composition according to claim 37 wherein

X_1 is absent or is W;

5

X_2 is absent or is R;

X_3 is P or K; and

X_4 is absent or is G or H.

39. A composition according to claim 38 wherein the peptide comprises the amino acid sequence N P F P T W R K R P G [SEQ ID NO:3].

10 40. A composition according to claim 38 wherein the peptide comprises the amino acid sequence N P F P T R K R P [SEQ ID NO:4].

41. A composition according to claim 38 wherein the peptide comprises the amino acid sequence N P F P T W R K R P [SEQ ID NO:5].

15 42. A composition according to claim 38 wherein the peptide comprises the amino acid sequence N P F P T W K R K H [SEQ ID NO:6].

43. A composition according to claim 37 wherein the analgesic properties of the peptide are inhibited by naloxone and/or L-NAME.

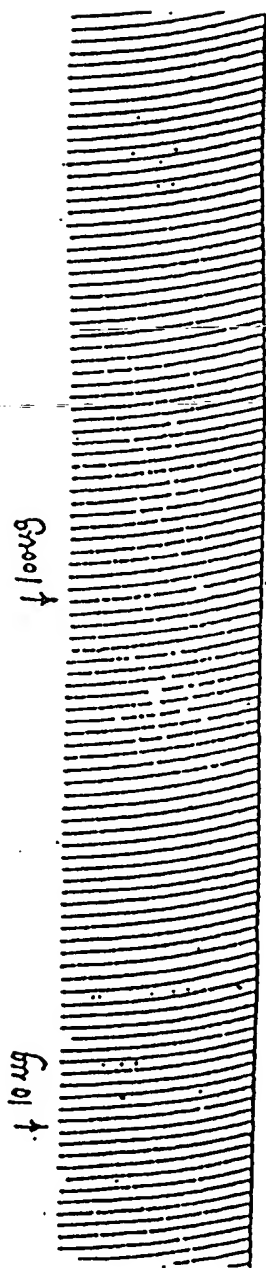


Fig. 1

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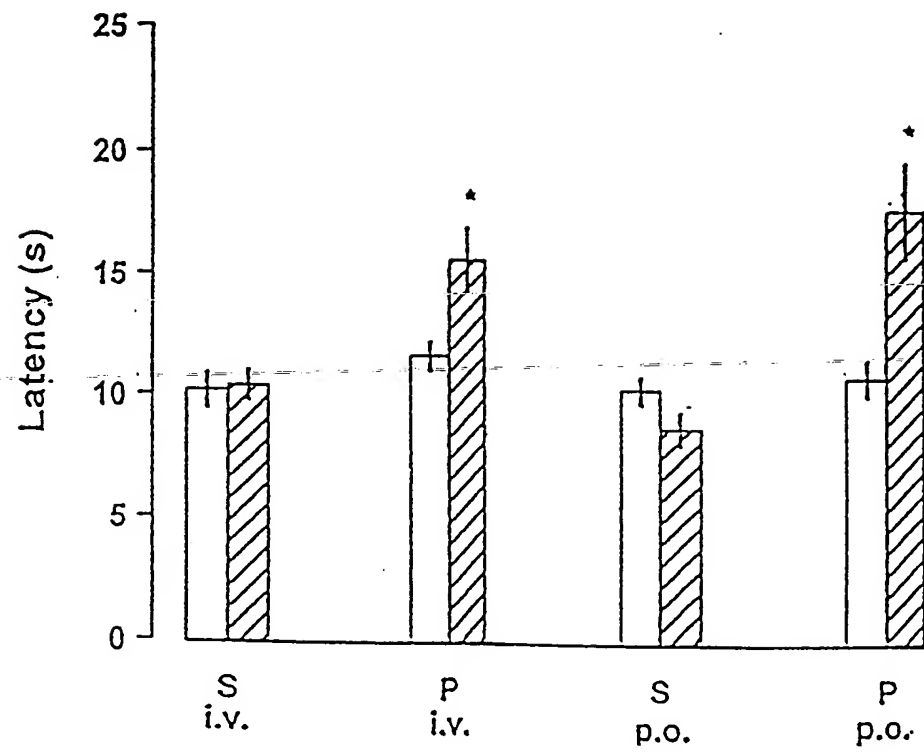


Fig. 2

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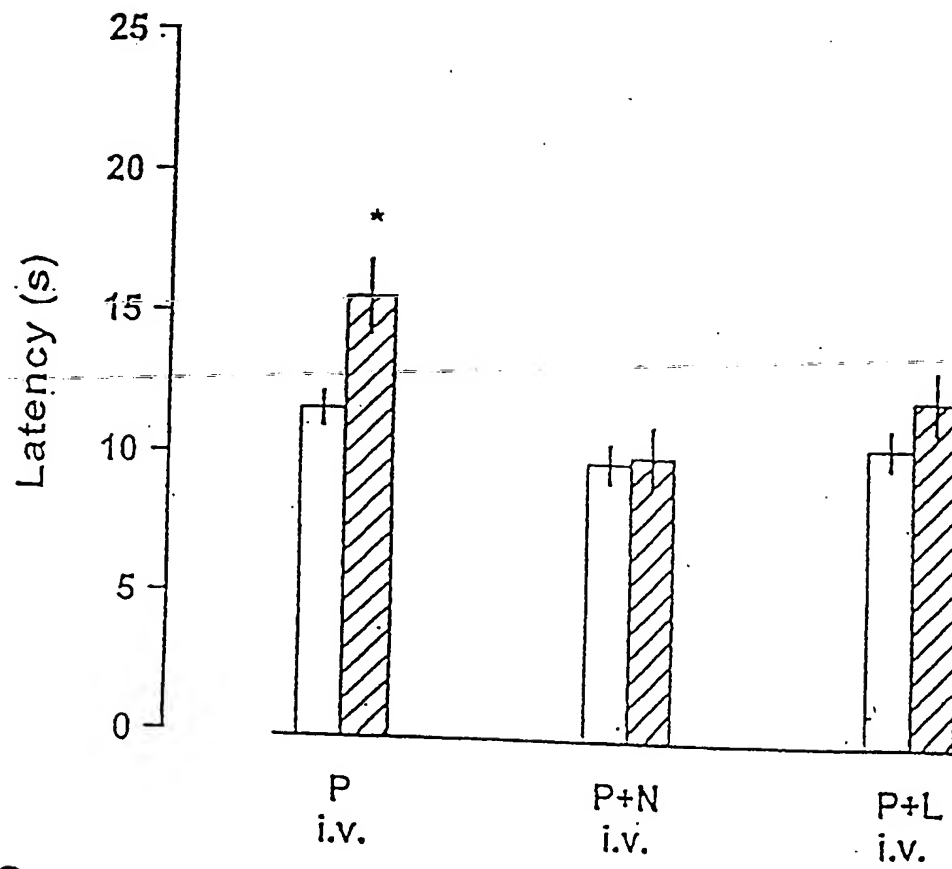


Fig. 3

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: NATIONAL UNIVERSITY OF SINGAPORE
- (ii) TITLE OF INVENTION: THERAPEUTIC MOLECULES
- 5 (iii) NUMBER OF SEQUENCES: 6
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: INDUSTRY & TECHNOLOGY RELATIONS OFFICE (INTRO)
 - (B) STREET: 10 KENT RIDGE CRESCENT
NATIONAL UNIVERSITY OF SINGAPORE
 - 10 (C) CITY: SINGAPORE
 - (D) STATE: SINGAPORE
 - (E) COUNTRY: SINGAPORE
 - (F) ZIP: 119260
- 15 (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- 20 (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- 25 (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: APPLIED RESEARCH CORPORATION
 - (C) REFERENCE/DOCKET NUMBER: PAP078-PCT
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: +65 7755822
 - (B) TELEFAX: +65 7730924
 - (C) E-MAIL: arc@pacific.net.sg

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Asn Pro Phe Pro Thr

5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Asn Pro Phe Pro Thr Xaa, Xaa, Lys Arg Xaa, Xaa,

5

10

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Asn Pro Phe Pro Thr Trp Arg Lys Arg Pro Gly

5

10

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Asn Pro Phe Pro Thr Arg Lys Arg Pro
5 9

5 (2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

10 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Asn Pro Phe Pro Thr Trp Arg Lys Arg Pro
5 10

15 (2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

20 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Asn Pro Phe Pro Thr Trp Lys Arg Lys His
5 10

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG 98/00087

A. CLASSIFICATION OF SUBJECT MATTER

IPC⁶: A 61 K 38/17, A 61 K 39/38

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC⁶: A 61 K 38/17, A 61 K 39/38

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, EPODOC, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 92/08 472 A1 (COR THERAPEUTICS, INC) 29 May 1992 (29.05.92), page 5, lines 1-9; page 11, lines 7-10; page 15, lines 7-24; table 1; claims 1-9.	1-12, 14-25, 27-42
A	DE 2 130 708 A (ABBOTT LABORATORIES) 23 December 1971 (23.12.71), claim 1.	27, 28
A	Database WPI on Questel, week 25, London, Derwent Publications Ltd., AN 83-32 708 K & JP 57-116 019 A (HORINOUCI S) 19 July 1982 (19.07.82) abstract.	1, 14, 29
A	Database WPI on Questel, week 32, London, Derwent Publications Ltd., AN 95-241 319 & CN 1 088 832 A (YIN Z) 06 July 1994 (06.07.94) abstract.	1, 14, 29
A	Database WPI on Questel, week 26, London, Derwent Publications Ltd., AN 97-281 564 & CN 1 102 570 A (CENT DEV ANIMAL'S VENOM & SNAKE SOURCE) 17 May 1995 (17.05.95) abstract.	1-3, 14-16, 29-33

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 21 January 1999 (21.01.99)	Date of mailing of the international search report 17 February 1999 (17.02.99)
Name and mailing address of the ISA/ AT Austrian Patent Office Kohlmarkt 8-10; A-1014 Vienna Facsimile No. 1/53424/535	Authorized officer Mosser Telephone No. 01/53424/435

INTERNATIONAL SEARCH REPORT

Internal application No.

PCT/SG 98/00087

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 14-26
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 14-26 concern a method of treatment of the human or animal body by therapy (see PCT Rule 39.1(iv)) the search was carried out and based on the alleged effects.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐

The additional search fees were accompanied by the applicant's protest.

☐

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/SG 98/00087

Im Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
WD A1 9208472	29-05-92	AU A1 90905/91	11-06-92
		AU B2 668079	26-04-96
		CA AA 2101759	17-05-92
		EP A1 557442	01-09-93
		EP A4 557442	05-10-94
		JP T2 6504765	02-06-94
		US A 5342830	30-08-94
		US A 5679542	21-10-97
		US A 5744584	28-04-98
DE A 2130708	23-12-71	FR A5 2104761	21-04-72
		GB A 1322671	11-07-73
		ZA A 7103774	26-01-72